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REGULATION OF OXIDATIVE PHOSPHORYLATION THROUGH PARALLEL ACTIVATION

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ABSTRACT

When the mechanical work intensity in muscle increases, the elevated ATP consumption rate must be matched by the rate of ATP production by oxidative phosphorylation in order to avoid a quick exhaustion of ATP. The traditional mechanism of the regulation of oxidative phosphorylation, namely the negative feedback involving [ADP] and [P_i] as regulatory signals, is not sufficient to account for various kinetic properties of the system in intact skeletal muscle and heart in vivo. Theoretical studies conducted using a dynamic computer model of oxidative phosphorylation developed previously strongly suggest the so-called each-step- activation (or parallel-activation) mechanism, due to which all oxidative phosphorylation complexes are directly activated by some cytosolic factor/mechanism related to muscle contraction in parallel with the activation of ATP usage and substrate dehydrogenation by calcium ions. The present polemic article reviews and discusses the growing evidence supporting this mechanism and compares it with alternative mechanisms proposed in the literature. It is concluded that only the each-step-activation mechanism is able to explain the rich set of various experimental results used as a reference for estimating the validity and applicability of particular mechanisms.

Keywords: metabolism regulation, energy metabolism modeling, each-step activation, skeletal muscle, heart

Introduction

To understand the dynamic behavior of some metabolic pathway/system it is not enough to know the scheme of enzymatic reactions entering into the composition of this pathway/system. It is necessary to know also how the system responds to some external physiological stimuli or disturbances. To accomplish this one needs to integrate all relevant quantitative aspects of the system (e.g., enzyme kinetics, metabolite and external effector concentrations) into one whole. Because the human brain is not adopted to such kind of analysis, dynamic computer models are appropriate tools for this purpose.

Oxidative phosphorylation is the main process responsible for ATP production in most animal cells. The central mechanism responsible for oxidative ATP synthesis is known since Mitchell published his chemiosmotic theory [1] due to which the proton gradient across the inner mitochondrial membrane is the key intermediate in transferring the Gibbs free energy from the redox span of the respiratory chain to the phosphorylation potential. The general scheme of the oxidative phosphorylation system in muscle is presented in Fig. 1.

The demand for ATP in the cell may vary depending on physiological conditions. The main physiological extracellular regulatory stimuli that modulate the rate of ATP turnover are different hormones (e.g., adrenaline, glucagon, vasopressin) in non-excitable tissues (e.g., liver) and neural stimulation in excitable tissues (e.g., skeletal muscle and heart). Heart is additionally stimulated by hormones and by preload pressure according to the Frank-Starling mechanism [2]. The secondary cytosolic messengers that transfer the external signal to different processes involved in ATP consumption and production are Ca^{2+}, cAMP and perhaps other, still undiscovered factors.

The dynamic response of the energetic system of the cell to elevated work is one of the most important aspects of cell functioning. In particular, it is important to know how the ATP production by oxidative phosphorylation is regulated in response to an elevated energy demand. Appropriate computer models can be a very useful research tool for studying this problem.

The present article is intended to put together, overview, systematize and integrate different arguments supporting the so-called parallel activation (each-step-activation) mechanism of the regulation of oxidative phosphorylation in response to varying energy demand, formulated during last several years by the author and other researches. Many of these arguments are dispersed among

other (mostly research) papers and some of them are first discussed in the present paper. A special emphasize is put on oxidative phosphorylation in intact skeletal muscle and heart. Five different mechanisms of the regulation of oxidative phosphorylation proposed in the literature are confronted with a broad range of various experimental data. The arguments and discussion appearing in the present article have never before been presented in one paper, and therefore the present article summarizes the author's opinions concerning the regulation of oxidative phosphorylation during work transitions in intact tissues. The article intentionally constitutes a combination of a review and a polemic paper, because, in the author's opinion, this way of presentation is optimal for summarizing the arguments supporting the each-step-activation mechanism.

Regulation of oxidative phosphorylation in muscle – possible mechanisms

In working muscle ATP is mostly used by actomyosin-ATPase, Ca^{2+}-ATPase and, in heart, Na^+/K^+-ATPase. Oxidative phosphorylation in mitochondria is the main source of ATP in most muscles under most physiological conditions. During rest-to-work transitions in skeletal muscle and low-to-high work transitions in heart a substantial increase in energy demand (ATP usage) may take place (in skeletal muscle the energy demand may increase even over 100 times). The rate of ATP synthesis must match very quickly the rate of ATP utilization in order to avoid a very rapid ATP exhaustion, termination of exercise or even cell death. Different mechanisms responsible for adjusting the rate of ATP production by oxidative phosphorylation to the current energy demand have been postulated in the literature.

Due to the first mechanism, that was originally proposed by Chance and Williams on the basis of their studies of the isolated mitochondria system [3,4], only ATP usage (output of the system) is directly activated by calcium ions during elevated muscle work, while oxidative phosphorylation is activated only indirectly, through a negative feedback involving an increase in [ADP] (and $[P_i]$). This mechanism will be called here the output-activation mechanism (Mechanism A). The dependence of VO_2 on [ADP] measured by Chance and Williams (and afterwards by many other authors) was hyperbolic (first-order at low ADP concentrations). Jeneson and co-workers [5] modified this mechanism and postulated that the mechanistic VO_2-[ADP] dependence is actually much steeper, at least second order (this modified output-activation mechanism can be called the mechanistic-ultrasensitivity mechanism, Mechanism D). Finally, Saks and co-workers [6-8] supplemented the original output-activation mechanism with the assumption that in intact muscle there exist significant ADP diffusion gradients and therefore creatine kinase (CK) is significantly displaced from thermodynamic equilibrium, what has a significant impact on the kinetic properties of oxidative phosphorylation (let us call this mechanism the CK-disequilibrium mechanism, Mechanism E).

The discovery of the activation by calcium ions of the three ‘key’ (flux-controlling) TCA (tricarboxic acid) cycle dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase) prompted several authors to postulate that substrate dehydrogenation (input of the system) is directly activated in parallel with ATP usage (input/output-activation mechanism, Mechanism B) during rest-to-work or low-to-high work transition [9,10]. However, due to

this mechanism oxidative phosphorylation is still activated only indirectly, by an increase in [ADP] and NADH/NAD^+ ratio.

Theoretical studies by means of the computer model of oxidative phosphorylation in skeletal muscle mitochondria, intact skeletal muscle and intact heart developed previously [11-14] led to the conclusion that some cytosolic factor/mechanism X activates directly all oxidative phosphorylation complexes (complex I, complex III, complex IV, ATP synthase, ATP/ADP carrier, phosphate carrier) in parallel with the activation of ATP usage and substrate dehydrogenation [12,15,16,13,17,14,18]. This mechanism has been named the each-step-activation mechanism or parallel-activation mechanism (Mechanism C) [12]. Within this mechanism the feedback regulation via [ADP] and [P_i] plays only a minor, fine-tuning role in intact skeletal muscle in vivo [12,15,16,13,17], and essentially no role in intact cardiac muscle in vivo [14,18].

Of course, the Mechanism C (each-step activation) and Mechanism B (input/output activation) may seem similar in this that in both cases a direct activation of ATP usage and ATP production takes place. However, as it is discussed below, they differ very much in the kinetic respect. Generally speaking, the increase in the flux through the oxidative phosphorylation system during a rest-to-work or low-to-high work transition may be potentially caused by three (groups of) factors: (a) increase in [ADP] and [P_i] (decrease in the phosphorylation potential), (b) increase in the NADH/NAD^+ ratio and/or (c) direct activation of (particular enzymes of) oxidative phosphorylation by some cytosolic factor related to muscle contraction. The increase in the flux brought about by changes in intermediate metabolite concentrations (factors a and b) is a consequence of two quantitative parameters: (i) physiological changes in metabolite concentrations during a rest-to-work transition (or low-to-high work transition) and (ii) sensitivity of the oxidative phosphorylation system to particular metabolite concentrations (quantified within Metabolic Control Analysis by the so-called elasticity coefficients, see e.g. [26]).

Historically, a direct parallel activation of at least ATP usage, substrate dehydrogenation and ATP/ADP carrier was first proposed in 1992 by Korzeniewski and Froncisz [20] in order to explain the constancy of ATP/ADP, of cytochrome c reduction level and of NAD reduction level, and the decrease of the flux control coefficient of ATP/ADP carrier during stimulation of hepatocytes by Ca^{2+}-related hormones (vasopressin, glucagon, adrenaline); a computer model of oxidative phosphorylation in hepatocytes was used in those studies. A few months later in 1992, and in 1994, Hochachka and

Matheson postulated that some enzymes in the ATP-producing system (they did not specify which ones) are ‘latent’ in resting skeletal muscle and are activated during muscle contraction [21,22]. In 1995 Korzeniewski and co-workers postulated, using the so-called Proportional Activation Approach, that ∆p-producing and ∆p-consuming system as well as ATP-producing and ATP-consuming system are activated to a very similar extent during activation of hepatocytes by vasopressin and during neural stimulation of skeletal muscle and heart, respectively [23]. In the same year Fell and Thomas formulated, in an abstract and general way, their ‘multi-site modulation’ idea [24,25] due to which the activities of many enzymes must be directly modulated in order to cause large changes in flux and at the same time to keep intermediate metabolite concentrations relatively unchanged. In 1998 Korzeniewski postulated [12], using the computer model of oxidative phosphorylation in muscle mitochondria [11], that all oxidative phosphorylation complexes must be directly activated to a large extent during skeletal muscle contraction, and introduced the name ‘parallel activation’. Next, several theoretical computer-aided studies based on the model of oxidative phosphorylation in intact skeletal muscle and heart supplied much more evidences supporting the parallel-activation (or each-step-activation) mechanism [12-18].

Dynamic computer model of oxidative phosphorylation

The theoretical studies leading to the concept of each-step activation were performed using the previously-developed computer model of oxidative phosphorylation in isolated mitochondria [11,12], intact skeletal muscle [13] and intact heart [14]. The following enzymes/processes/metabolic blocks (shown in Fig. 1) are taken into account explicitly within the model: substrate dehydrogenation (hydrogen supply to the respiratory chain including TCA cycle, glycolysis, glycogenolysis, glucose transport, fatty acid β-oxidation, fatty acid transport and so on), complex I, complex III, complex IV (cytochrome c oxidase), proton leak, ATP synthase, ATP/ADP carrier, phosphate carrier, adenylate kinase, creatine kinase, ATP usage (actomyosin-ATPase, Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase, basal ATP consumption). The dependences of the rates of particular reactions/processes/metabolic blocks on different metabolite concentrations are described by appropriate kinetic equations. For instance, the rate of the reaction catalyzed by cytochrome oxidase (complex IV) v_{C4} is expressed as follows:

$$v_{C4} = k_{C4} \cdot a^{2+} \cdot c^{2+} \frac{1}{1 + \frac{K_{mO}}{O_2}} \quad (1)$$

where k_{C4} is the rate constant, K_{mO} is the mechanistic Michaelis-Menten constant of cytochrome oxidase for oxygen, and a^{2+} and c^{2+} are concentrations of the reduced forms of cytochrome a_3 and cytochrome c, respectively. The time variations of the metabolite concentrations which constitute state variables (NADH, ubiquinol, reduced form of cytochrome c, O_2, internal protons, internal ATP, internal P_i, external ATP, external ADP, external P_i, external protons, PCr) are expressed in the form of a set of ordinary differential equations. For instance, the differential equation for the reduced form of cytochrome c has the following form:

$$d\,c^{2+}/dt = \left(v_{C3} - 2 \cdot v_{C4}\right) \cdot 2 \cdot R_{cm} \quad (2)$$

where the rate of change of the concentration of the reduced form of cytochrome c in time dc^{2+}/dt is equal to the difference between the rate of its production by complex III (v_{C3}) and the rate of its consumption by complex IV (v_{C4}) (described by Equ. 1); 2 and R_{cm} describe the stoichiometry (2 electrons for 1 NADH, 4 electrons for 1 O_2) and the ratio of cell volume to mitochondria volume, respectively. Of course, in a steady-state no changes in metabolite concentrations take place, because the rates of production and consumption of each metabolite are exactly equal. The other

(output) variable values (other metabolite concentrations, thermodynamic forces) are calculated from the independent variable values. The set of differential equations is integrated numerically. In each iteration step, new values of rates, concentrations and other parameters are calculated on the basis of the corresponding values from the previous step. The Gear procedure is used for numerical integration and the simulation programs are written in the FORTRAN programming language. The complete description of the model is located on the web site http://awe.mol.uj.edu.pl/~benio/ .Within the model a direct n-fold activation of some enzyme/process is equivalent to the n-fold increase in the rate constant or maximal velocity of this enzyme/process.

Only a computer model that is very well tested by comparison with a possibly broad set of different parameter values and system properties encountered in experimental studies may be used for reliable theoretical studies on the modeled metabolic system and for predicting the existence of new phenomena. The discussed computer model has been extensively tested for a very broad set of different properties of the oxidative phosphorylation system [13-18,26-30]. The validation of the model concerns oxidative phosphorylation in isolated mitochondria, isolated hepatocytes, intact skeletal muscle and intact heart. The correctly predicted system properties include, among others: (1) the values of fluxes (oxygen consumption and ATP turnover) and concentrations of metabolites (ADP, ATP, P_i, PCr, Cr, $NADH/NAD^+$, reduction level of cytochrome c, reduction level of cytochrome a_3, Δp – protonmotive force and O_2) in different steady-states imposed by various energy demands and oxygen concentrations; (2) changes over time of fluxes and metabolite concentrations during transitions between different steady-states (rest-to-work transition, aerobiosis-to-anaerobiosis transition); (3) the values of the flux control coefficients (defined within Metabolic Control Analysis) quantifying the control of particular enzymes, processes, and metabolic blocks over the oxygen consumption flux in different steady-states; (4) the dependence of the respiration rate on the activities of different enzymes, obtained by the titration of particular oxidative phosphorylation complexes with specific inhibitors.

Some kinetic phenomena explained by each-step activation

The each-step-activation mechanism is able to account for many various, apparently independent kinetic properties of the oxidative phosphorylation system in different tissues. Some of these properties, concerning mostly intact skeletal muscle and heart in vivo, are discussed below.

Steep phenomenological VO_2-[ADP] relationship

In most experiments on isolated mitochondria the mechanistic VO_2-[ADP] relationship is near-hyperbolic (first-order at low [ADP]) (see e.g. [31-33]). On the other hand, the phenomenological VO_2-[ADP] relationship encountered in intact skeletal muscle during rest-to-work transition is usually much steeper (at least second-order) – a large relative increase in VO_2 is accompanied by only a moderate increase in [Cr], [P_i] and free [ADP] (calculated from creatine kinase equilibrium) [22,5,34-41]: compare Table 1. In intact heart in vivo during low-to-high work transition a five-fold increase in VO_2 and work intensity takes place without any significant changes in [PCr], [ATP], [P_i] and total (free and protein-bound) [ADP]; also the calculated free [ADP] is essentially constant [42-44] (compare last row in Table 1). Therefore in the last case the phenomenological reaction order is apparently infinite. Such a kinetic behavior of the system can be explained by a high-intensity each-step-activation in skeletal muscle [12,15,45] and perfectly-balanced each-step activation in heart [14,18]. Because the control over the oxygen consumption flux (and ATP turnover flux) is distributed more or less uniformly between particular oxidative phosphorylation complexes (the flux control coefficients for particular complexes are of the same order of magnitude, none of them is very close to zero) [46,11], all steps must be directly activated by some cytosolic factor X in order to cause a great increase in the flux and at the same time to keep intermediate metabolite concentrations relatively constant [12,45,14]. The general scheme of the phenomenological VO_2-[ADP] relationship in different skeletal muscles under various conditions and in intact heart in vivo are presented in Fig. 2 and Fig. 3, respectively.

Maximal VO_2 in skeletal muscle

Tonkonogi and Sahlin paid attention to the fact that the maximal VO_2 in intact human skeletal muscle is, when recalculated for the amount of mitochondria, 2-5 times greater than the maximal VO_2 in isolated mitochondria, skinned fibers and muscle homogenate [47]. While the maximal VO_2 in

isolated mitochondria corresponds to about 3-4 mM/min (70-90 ml/kg/min) [47] in skeletal muscle, the maximal oxygen consumption in intact muscle equals 16 mM/min (350 ml/kg/min) [47-49,13] or even 26 mM/min (580 ml/kg/min) [50] during single muscle exercise and is about half during whole body exercise [51,47,13] (1 mmol = 22.4 ml; recalculation of molar amount of oxygen for oxygen volume under normal conditions). This is exactly what should be expected if it is assumed that intensive parallel activation of different oxidative phosphorylation complexes takes place during rest-to-work transition in intact skeletal muscle [12,16,45]. This property of the system can be also seen in Fig. 2 when the curve for high parallel activation is compared with the curve for no parallel activation. A similar situation can be expected in intact heart in vivo – see Fig. 3. However, in this case the experimental evidence is not so clear – compare the discussion below.

Relative constancy of NADH/NAD$^+$

During rest-to-work transition in skeletal muscle [NADH] may either decrease [52,53] or increase [54,55], while during transition to a higher work in intact heart in vivo it remains essentially constant [56,44]. This kinetic behavior of the system can be easily reproduced by the computer model of oxidative phosphorylation if it is assumed that there is a subtle balance of the direct activation of NADH production and NADH consumption: if NADH consumption is activated slightly more than NADH production then [NADH] decreases, in the opposite case [NADH] increases and if both NADH production and NADH consumption are activated to exactly the same extent [NADH] does not change.

PCr on/off asymmetry

In perfused glycolytic skeletal muscle stimulated electrically the time course of [PCr] during on-transient (rest-to-work transition) and off-transient (work-to-rest transition) is symmetrical – the half-transition time $t_{1/2}$ for the near-exponential decrease/increase in [PCr] is the same and equals about 80-90 seconds [57]. It seems that in intact oxidative skeletal muscle stimulated neurally the on/off PCr transient is symmetrical in some cases ([58], estimated from the symmetry of the VO_2 on/off transient) and asymmetrical in other cases – [PCr] decreases faster during on-transient than increases during off-transient [59,60] (this asymmetry is clearly significant, although no statistical analysis has been performed). As it is discussed below and in [15], it is likely that in electrically stimulated perfused glycolytic skeletal muscle there is no each-step activation and therefore only the output-activation

mechanism prevails. Therefore there is no reason for the PCr on/off asymmetry to take place. On the other hand, it was postulated [15] that in working intact oxidative skeletal muscle in vivo the oxidative phosphorylation complexes are directly activated by some cytosolic factor X and therefore [PCr] decreases faster at the onset of exercise than increases after the termination of exercise, when the direct activation of oxidative phosphorylation is turned off. This is equivalent to the PCr on/off asymmetry. However, as it is discussed elsewhere [15], it is likely that in many cases the direct activation of oxidative phosphorylation is not turned off instantly after the termination of exercise, but it decays in the exponential way (as a result of e.g. protein dephosphorylation or calcium transport to mitochondria [61]) according to the following equation:

$$m = 1 + (n-1) \cdot e^{-t/\tau(OFF)} \qquad (3)$$

where m is the current (at time t) relative activity of oxidative phosphorylation expressed as a multiple of the resting activity, t is the time after the onset of off-transition, n is the relative activity of oxidative phosphorylation at the high-work steady-state expressed as a multiplicity of the resting activity, while τ(OFF) is the characteristic decay time of inactivation of oxidative phosphorylation after the termination of exercise. As it can be seen in Fig. 4, when τ(OFF) = 0 the half-transition time $t_{1/2}$ equals about 25 s for on-transient and about 80 s for off-transient. When τ(OFF) equals about 100 s the PCr on/off transition is symmetrical even in intact oxidative skeletal muscle in vivo – $t_{1/2}$ for both on- and off-transient equals about 25 s. When the characteristic decay time has an intermediate value between 0 s and 100 s, say 30 s, $t_{1/2}$ for the off-transient has also an intermediate value between 80 s and 25 s, in this case – 40 s (not shown). In fact, this moderate 25 s/40 s PCr on/off asymmetry seems to be more physiologically relevant than the large 25 s/80 s asymmetry simulated for τ(OFF) = 0 s [59,60]. Therefore, it seems that in intact oxidative skeletal muscle τ(OFF) is always significantly greater than 0 s. Generally, the discussed theoretical predictions agree well with experimental data where the PCr on/off asymmetry was observed in some experimental studies on oxidative skeletal muscle in vivo [69,60], while in some other studies [58] the PCr on/off symmetry is likely to take place. The above discussion leads to the conclusion that the maximal rate of PCr resynthesis during muscle recovery in oxidative skeletal muscle is not a measure of the maximal capacity of oxidative phosphorylation for ATP synthesis, as proposed by several authors.

PCr recovery overshoot

If the characteristic decay time of inactivation of oxidative phosphorylation during off-transient τ(OFF) equals 300 s or more the phenomenon called the PCr recovery overshoot [62,63] appears – after the termination of exercise [PCr] rises above its resting value and then slowly approaches the resting level – see Fig. 4, compare also [15,64,39]. The kinetic background of the PCr recovery overshoot is explained in Fig. 5. The activity (rate constant) of ATP usage increases almost instantly several times (in this simulation: 50 times) at the onset of exercise and decreases almost instantly to the resting value after the termination of exercise. On the other hand, the activity of ATP production by oxidative phosphorylation (rate constants of all oxidative phosphorylation complexes) is (are) elevated (in this simulation: almost 6 times) very quickly at the onset of exercise, but decrease(s) slowly (in this simulation: with the characteristic decay time of 300 s) after the termination of exercise. Therefore in the early period of muscle recovery ATP usage is as active as at rest, while ATP production is much more active than at rest. A direct consequence of this kinetic property of the system is a transient overshoot in [PCr] and phosphorylation potential, and undershoot in [ADP] and [P_i] [15,64].

Summing up, τ(OFF) < ~100 gives PCr on/off asymmetry, τ(OFF) = ~100 gives PCr on/off symmetry, and τ(OFF) > ~200 gives PCr recovery overshoot.

VO_2 and PCr on-kinetics

During rest-to-work transition both pulmonary and muscle VO_2 increases and muscle [PCr] decreases in a near-exponential way [65,59]. Depending on the training and health status the half-transition time $t_{1/2}$ for VO_2 and [PCr] equals about 20 – 65 s in human locomotory muscles during voluntary exercise [65]. In glycolytic skeletal muscle stimulated electrically $t_{1/2}$ for VO_2 and [PCr] is longer and equals 80-90 s [57]. These kinetic properties of the system can be accounted for by the discussed model of oxidative phosphorylation when it is assumed that the intensity of each-step activation is moderate-to-high in intact skeletal muscle stimulated neurally and low or zero in glycolytic skeletal muscle stimulated electrically [15]. The exponentiality of the time course of VO_2 and [PCr] at the onset of exercise is not trivial and is not predicted by the mechanistic-ultrasensitivity mechanism (Mechanism D) and CK-disequilibrium mechanism (Mechanism E), as discussed below and in [30].

Training-induced adaptation of oxidative phosphorylation

The physical training causes an acceleration of the pulmonary VO_2 kinetics at the onset of moderate-intensity exercise [66,67] and an improvement in the ADP and/or PCr homeostasis (a smaller increase in [ADP]/ decrease in [PCr] at a given increase in VO_2 and/or work intensity during rest-to-work transition) [68,69,34]. The shortening of the VO_2 transition time may be potentially caused by at least two factors [29]: the training-induced increase in the amount of mitochondria encountered in experimental studies [70,71] and the increase in the intensity of the each-step activation. However, only the latter phenomenon may account for the significantly elevated ADP homeostasis [29], especially at lower work intensities and in the case when muscle training does not lead to a decrease in resting free [ADP], and therefore the mechanism postulated by Gollnick and Saltin [29] does not work [73]). This homeostasis can be expressed as the slope of the phenomenological VO_2-[ADP] relationship [29] (the steeper the slope, the better the homeostasis):

$$R = \frac{VO_{2(work)} / VO_{2(rest)}}{[ADP]_{(work)} / [ADP]_{(rest)}} \quad (4)$$

At low work intensities where oxidative phosphorylation is not saturated with ADP, the ratio of R in trained muscle to R in untrained muscle ($R_{trained} / R_{untrained}$) is close to 1 for an increase in mitochondria / mitochondrial proteins, but greater than 1 for intensified parallel activation [29]. The available experimental data [68,69,34] give the value of this ratio between 1.6 and 3.1 [29], suggesting that the training-induced adaptation of oxidative phosphorylation in skeletal muscle involves an increase in the intensity of the each-step activation (in [34] the difference in the slope of the VO_2/ADP relationship at low work intensities between trained and untrained rats is much smaller when VO_2 is scaled for cytochrome c content; however, this does not prove that the increase in mitochondria content is fully responsible for the increase in metabolite stability, but only shows that the increase in mitochondria amount accompanies the increase in metabolite stability). The increase in the steepness of the phenomenological VO_2-[ADP] relationship caused by muscle training is indicated in Fig. 2 by a dotted arrow.

VO_2 per mg of cytochrome c at a given [ADP] in trained muscle and hypothyroid muscle

It has been demonstrated in experimental studies [34] that the respiration rate scaled for the amount of cytochrome c is significantly higher at a given ADP concentration in trained muscle than in untrained muscle and in control muscle than in hypothyroid muscle. This finding can be explained [15]

by the assumption that muscle training increases the intensity of each-step activation, while hypothyroidism decreases this intensity.

Variability of the kinetic properties of oxidative phosphorylation in different muscles

The kinetic properties of oxidative phosphorylation vary significantly between different experimental studies performed on different muscles in various experimental conditions [15]. In glycolytic skeletal muscle, perfused muscle and electrically-stimulated muscle the following set of properties tends to be observed: 1. shallow VO_2/ADP and VO_2/Cr relationships (relatively low increase in VO_2 at a given increase in [ADP] or in [Cr]); 2. long (over 60 s) half-transition time $t_{1/2}$ for VO_2 and PCr during on-transient and off-transient; 3. symmetry between the PCr on- and off-kinetics [62,39,35,22,57]. In oxidative skeletal muscle, muscle in situ and neurally-stimulated skeletal muscle the opposite set of properties is usually observed: 1. steep VO_2/ADP and VO_2/Cr relationships (high increase in VO_2 at a given increase in [ADP] or in [Cr]); 2. short (below 60 s) half-transition time $t_{1/2}$ for VO_2 and PCr during on-transient and off-transient; 3. asymmetry (in many cases, see above) between the PCr on- and off-kinetics [62,39,35,22,5,66,59,60,69]. The first set of properties is generated by the computer model of oxidative phosphorylation if a low or none each-step activation is assumed, while the second set is predicted when medium- or high-intensity each-step activation is involved [15]. Of course, all intermediate cases are also possible. Fig. 2 presents schematically the phenomenological VO_2-[ADP] relationship for high, moderate and no each-step activation. Additionally, different characteristic times of the decay during muscle recovery of the each-step activation are able to explain, as discussed above, why the PCr overshoot is present in some cases and absent in other cases, and why there can take place symmetry or asymmetry between PCr on- and off-transient.

In intact heart in vivo essentially no changes in calculated free [ADP] and other metabolite concentrations take place during low-to-high work transition [42,43,56,44] and the transitions between different work intensities are very quick [74]. These properties of the system can be explained by the computer model of oxidative phosphorylation if a perfectly balanced each-step activation is assumed in this case [14,18] (compare Fig. 3). Therefore, the idea of each-step activation enables to explain the great variability of the kinetic properties of oxidative phosphorylation encountered in different muscles and various experimental conditions.

Comparison of different mechanisms of regulation of oxidative phosphorylation

The only way to test the validity and applicability of a kinetic model of a metabolic pathway involving a particular regulatory mechanism is to confront the theoretical predictions generated by such a model with a possibly broad range of parameter values and properties of the modeled system encountered in experimental studies. Below some chosen experimental results are enumerated that will serve in the present article as a reference for the estimation of different mechanisms of the regulation of oxidative phosphorylation in muscle in response to an elevated energy demand.

Result 1. In intact skeletal muscle and heart in vivo a great relative increase in VO_2 is accompanied by a moderate (skeletal muscle) or no (statistically significant) (heart) increase in $[P_i]$ and [Cr] and calculated free [ADP] during rest-to-work or low-to-high-work transition (compare Table 1) [22,70,37-44,35,34,5].

Result 2. In intact human skeletal muscle the maximal VO_2 recalculated for the mitochondria volume is 2-5(-7) times greater than in isolated mitochondria [47] (see also [48-50] and calculations in [13]). It was postulated that the maximal VO_2 in heart [75] and in skeletal muscle in quadrupeds [76] matches well the maximal VO_2 in isolated heart mitochondia and skeletal muscle mitochondria, respectively; however, as it is discussed below, the interpretation of these results is not so strightforward.

Result 3. In most experimental studies the VO_2-[ADP] relationship in isolated mitochondria is hyperbolic (first order al low [ADP]) (see e.g. [31-33]) .

Result 4. $[P_i]$ in intact heart is essentially constant (or, at best, changes little) during low-to-high work transition [42] and equals roughly 1-3 mM [77,78] (compare also discussion in [14]). $[P_i]$ may be significantly different for different substrates and hormones present in the perfusion medium, but is in the millimolar range [77].

Result 5. In resting skeletal muscle, where there is no mechanical work, the ATP usage for ‘basic’ processes keeping the cell alive (protein synthesis, DNA/RNA synthesis, ion circulation) accounts for about 50 % of oxygen consumption; the remaining oxygen consumption is due to proton leak [79,80]. In slowly beating heart, where substantial amount of ATP is used for mechanical work, the relative contribution of ATP usage to oxygen consumption can be expected to be essentially higher (about, say, 80 % [18]). The relative increase in the respiration rate (4 - 6 times) is similar to the

relative increase (2 - 4 times) in the rate-pressure product (proportional to the ATP usage for mechanical work) during low-to-high work transition in intact heart in vivo [42]. This means that the contribution of the proton leak to VO_2 is small even at low work – otherwise the relative increase in the rate-pressure product would be much greater than the relative increase in oxygen consumption. In fact, it was reported by Challoner that 84 % of the in vivo oxygen consumption was inhibited by arresting the heart [81]; it was estimated on the basis of those data that the contribution of the proton leak to oxygen consumption in working heart is, roughly, about 10 % [82].

Result 6. The $NADH/NAD^+$ ratio may either increase [54,55] or decrease [52,53] in skeletal muscle during rest-to-work transition and is essentially constant in heart during transition to a higher work [56,44].

Result 7. In some cases there is a PCr on/off asymmetry in skeletal muscle – the increase in [PCr] after the termination of exercise is slower than the decrease in [PCr] after the onset of exercise [59,60] (this asymmetry is clearly significant, although no statistical analysis has been performed).

Result 8. In some cases there is a PCr recovery overshoot in skeletal muscle – after the termination of exercise [PCr] increases over its resting level and then slowly approaches the resting value [62,51,39].

Result 9. The VO_2 and PCr on-kinetics in skeletal muscle is near exponential – oxygen consumption increases in the exponential way after the onset of exercise [65,59].

Result 10. The half-transition time $t_{1/2}$ for VO_2 and PCr during on-transient and off-transient in intact skeletal muscle in vivo is relatively short and equals 20-65 s [65].

Result 11. Muscle training improves the ATP/ADP homeostasis [68,69,34].

Result 12. VO_2 scaled for the amount of cytochrome c is greater at a given [ADP] in trained muscle than in untrained muscle and in control muscle than in hypothyroid muscle [34].

Result 13. The Δp-producing subsystem is little sensitive to Δp [80] and therefore the ATP-producing subsystem (containing the Δp-producing subsystem) cannot be very sensitive to ATP/ADP. This is because changes in ATP/ADP can affect the Δp-producing subsystem only via changes in Δp.

Result 14. There is a great diversity of the kinetic properties of oxidative phosphorylation in different muscles (glycolytic skeletal muscle, oxidative skeletal muscle, heart) and different experimental conditions (perfused muscle vs. muscle in vivo, electrically stimulated muscle vs. neurally stimulated muscle), comprising different slopes of the phenomenological VO_2-[ADP]

relationship, different half-transition times for VO_2 and [PCr], PCr symmetry/asymmetry between on-transient and off transient, and absence/presence of the PCr recovery overshoot (see above).

Result 15. In intact heart in vivo under physiological conditions the transitions between different work intensities are very quick. Under 'normal' physiological conditions (37 °C, normoxia, lack of inhibitors) the half-transition time $t_{1/2}$ for VO_2 equals 4-8(-11) s (see [74], Table 2), while for [PCr] can be as low as 2.5 s [74]. In isolated trabeculae a significant NADH undershoot/overshoot during low-to-high/high-to low work transition, respectively, takes place [83,84].

Result 16. The protonmotive force (Δp) does not change, or even slightly increases, during rest-to-work transition in skeletal muscle [80].

In Table 2 the five mechanisms of the regulation of oxidative phosphorylation mentioned above, namely: (A) output activation, (B) input/output activation, (C) each-step activation, (D) mechanistic ultrasensitivity and (E) CK disequilibrium, are confronted with the 16 experimental results presented above. It is indicated for each mechanism and result whether a given mechanism is able to account for a given result or not. Below, the confrontation of the mechanisms with particular results is discussed in more detail.

Mechanism A assumes that both mechanistic and phenomenological VO_2-[ADP] relationship is hyperbolic (first order at low [ADP]) [31-33] and therefore for obvious reasons this mechanism cannot account for Result 1. Substrate dehydrogenation has a low control over VO_2 [46,11] and therefore its direct activation cannot change significantly the phenomenological VO_2-[ADP] relationship; this implies that also Mechanism B cannot explain Result 1. Computer simulations demonstrated that Mechanism C can explain Result 1 (see above). Mechanism D is able in principle to account for the steep phenomenological VO_2-[ADP] relationship in skeletal muscle, but is helpless in the case of intact heart where this relationship is of essentially infinite order. Finally, Mechanism E can approximately account for Result 1 in heart [6] only under the assumption that oxidative phosphorylation in heart at low workload is in (or near) state 4 and therefore no ATP production takes place (what severely contradicts Result 5) and that [P_i] at low workload equals a few μM and increases by three orders of magnitude during transition to high work [6] (what contradicts Result 4). In fact, the relative constancy of [ADP] predicted by Mechanism E [6,7] has little to do with compartmentalized energy transfer and is based on the fact that in heart, because of a smaller total phosphate concentration than in e.g. skeletal muscle, just [P_i], and not [ADP] is limiting for the flux in the near

vicinity of state 4 (compare Fig. 3, the left end of the phenomenological VO_2-[ADP] relationship for no parallel activation; see [14] for a detailed discussion). However, as it is discussed above and below, the assumption that at low work heart is in (near) state 4 is completely unphysiological.

For obvious reasons Result 2 cannot be accounted for by mechanisms A, D and E, in which no direct activation of ATP supply is assumed. Mechanism B cannot help much, because substrate dehydrogenation has a low control over VO_2 [46,11] and therefore its direct activation cannot increase significantly the activity of oxidative ATP production. On the other hand, Mechanism C remains in a good agreement with Result 2 [13,45].

Only Mechanism D is in contradiction with Result 3, although in some experiments a mechanistic VO_2-[ADP] relationship of about second order was encountered in isolated mitochondria [5] (see also the discussion below).

Mechanisms A, B, D and E cannot account for Result 4. In particular, if Mechanism E is supplemented with the assumption that oxidative phosphorylation in heart with low workload is in state 4, $[P_i]$ increases by three orders of magnitude between low and high workload, from a few μM to a few mM, what severely contradicts Result 4. Mechanism C is able to explain Result 4 [14,18].

Result 5 does not agree with Mechanism E supplemented with the assumption that slowly beating heart is in (or near) state 4, as discussed above. This assumption is necessary to explain the relative constancy of [ADP] in heart because VO_2 is very sensitive to $[P_i]$ only in the near vicinity of state 4 (see discussion in [14]). The relative increase (2 – 4 - fold) in the rate-pressure product (proportional to the ATP usage for mechanical work) is even smaller than the relative increase in the respiration rate (4 - 6 times) during low-to-high work transition in intact heart in vivo [42]. This means that even at low work most oxygen consumption is due to ATP usage and not to proton leak. If the contribution of proton leak to VO_2 at low work in heart was, say, 90 % (or more), then a 5-fold increase in ATP usage would cause an increase in VO_2 by 40 % (to 140 %: 90 % +5 * 10 %) (or less). Experimental studies on the arrested heart strongly suggest that the contribution of the proton leak to oxygen consumption in working heart is around 10 % [81,82], in a good agreement with the predictions of the computer model developed by Korzeniewski and co-workers [14].

When only ATP usage (and therefore only the NADH-consuming system) is directly activated the $NADH/NAD^+$ ratio must decrease. On the other hand, an appropriately balanced direct activation of NADH production and NADH consumption may cause that [NADH] will decrease, increase or remain

unchanged (as discussed above). Therefore Result 6 can be accounted for by both Mechanism B and Mechanism C [16,14,18].

Only Mechanism C can explain the PCr on/off asymmetry (Result 7), because only within this mechanism oxidative phosphorylation is much more active during skeletal muscle work than during recovery (see above) [15].

Similarly, only Mechanism C can explain the PCr recovery overshoot (Result 8), because only within this mechanism oxidative phosphorylation can be more active during muscle recovery after exercise than at rest (see above) [15].

Mechanisms A, B and C predict a near-exponential VO_2 and PCr on-kinetics (a good agreement with Result 9) [30], while for Mechanisms D and E this kinetics is strongly non-exponential (contradiction with result 9) [30].

Computer simulations show that without each-step activation the half-transition time for VO_2 and [PCr] during on-transient and off-transient is long (80-90 s) [15]. This is what was found by Meyer in the case of glycolytic skeletal muscle stimulated electrically [57]. This experimental finding can be therefore explained within Mechanism A (and also Mechanism B that gives similar predictions in this case, not shown), in accordance with the proposal that there is no each-step activation in electrically stimulated glycolytic muscle [15] (see the discussion above). On the other, in neurally-stimulated skeletal muscle in vivo (voluntary exercise) the half-transition transition time for VO_2 and [PCr] is much shorter and equals 20-65 s [65]. A short $t_{1/2}$ is predicted by Mechanisms C, D and E [30].

Only Mechanism C can account for the training-induced increase in the phenomenological VO_2-[ADP] relationship (Result 11) [30]. Within Mechanism E it is able to modify the phenomenological VO_2-[ADP] relationship by modifying ADP diffusion limitations [7], but only under assumptions that contradict Results 4 and 5, as discussed above in the context of Result 1.

Result 12 constitutes the most direct manifestation of Mechanism C: this result means that oxidative phosphorylation during work is more active in trained muscle than in untrained muscle and in control muscle than in hypothyroid muscle. As discussed above (Results 1 and 2), substrate dehydrogenation has a too low control over the oxygen consumption flux to cause a significant increase in VO_2 when this metabolic subsystem is directly activated.

Mechanisms D and E are based on the assumption that the ATP-producing subsystem is very sensitive to [ADP] (because of a high mechanistic sensitivity in the case of Mechanism D and very low

P_i at low work in heart in the case of Mechanism E). This requires that Δp-producing system, being a part of the ATP-producing system, is very sensitive to Δp. Therefore Result 13 cannot be reconciled with mechanisms D and E.

Only the each-step-activation mechanism (Mechanism C) can account for the variability of the kinetic properties of oxidative phosphorylation in different muscles and various experimental conditions (Result 14), as discussed above. For instance, an increase in the each-step activation can explain the increase in the ADP homeostasis in heart during sheep development [85]. Mechanisms A, B and D predict that the kinetic properties of oxidative phosphorylation are the same in different situations. As discussed above, Mechanism E is able to modify the phenomenological VO_2-[ADP] relationship by modifying ADP diffusion limitations [7], but only under assumptions that contradict Results 4 and 5. It is unlikely that the diversity of the kinetic properties of oxidative phosphorylation in different muscles (Results 14 and 6) results from differences in the relative amounts/activities of oxidative phosphorylation complexes and substrate dehydrogenation subsystem, because such differences would mostly affect steady-state fluxes and metabolite concentrations and not the relative changes in fluxes and metabolite concentrations during transitions between different steady-states.

As it was shown previously [18], only Mechanism C is able to account for a significant increase/decrease in VO_2, for NADH undershoot/overshoot and for a very short transition time for VO_2 and [PCr] during low-to-high/high-to-low work transition in heart (Result 15).

The constancy (or even a small increase) of Δp during rest-to-work transition in skeletal muscle (Result 16) means that both Δp production and Δp consumption is directly activated during muscle work. Therefore Result 16 excludes Mechanisms A, D and E. Mechanism C is in a good agreement with this experimental result, while Mechanism B can explain it only for relatively low work intensities.

Table 2 and the above discussion clearly show that while Mechanisms A, B ,D and E may explain some single experimental results, only Mechanism C (each-step activation) is able to account for all the Results 1 – 16. Therefore this mechanism seems to be decidedly most relevant for the regulation of oxidative phosphorylation in intact skeletal muscle and heart.

It has been postulated that elevated extramitochondrial inorganic phosphate (P_i) concentration activates directly the complex III of the respiratory chain; this conclusion was based on the observation that an increased [P_i] increases the membrane electrical potential of the inner mitochondrial

membrane ($\Delta\Psi$) and the reduction level of complex III [86]. However, first, the total (extra- and intramitochondrial) [P_i] is essentially constant in intact heart in vivo and therefore cannot be a major regulatory signal for oxidative phosphorylation [42]. Second, the effect attributed to [P_i] in [86] could be in fact caused by a change in the contribution of ΔpH (pH gradient) to the protonmotive force (Δp) across the inner mitochondrial membrane at higher P_i concentrations. In the cited study the pH gradient was not measured directly, e.g., using a weak acid gradient; instead, the extramitochondrial pH was simply fixed by an appropriate buffer and the intramitochondrial pH was measured. However, in such a case a small systematic error in internal pH could result in a great relative error in ΔpH. This could be the reason why the pH gradient was very close to zero in the cited study, while most measurements give the value of ΔpH equal to about 30 mV (0.5 pH units) [87,88,89,90]; this value may differ by up to 10-20 % between different physiological energetic states. Therefore, an alternative explanation of the experimental results obtained in [86] is that increased [P_i] lowers ΔpH and rises $\Delta\Psi$ (while the sum of these potentials, Δp, slightly decreases) because at very low [P_i] the phosphate carrier, catalyzing the co-transport of P_i and H^+, works very slowly and therefore ΔpH is very high. This is exactly what was observed by Nicholls in his classical study [87], where ΔpH was measured by ion distribution (see Fig. 4 therein). A similar pattern of behavior was encountered in [89], although in this case $\Delta\Psi$ decreased as [P_i] increased. Complex III may be differently regulated by $\Delta\Psi$ and ΔpH and therefore the redox level of this complex increases. Third, the postulated mechanism constitutes in fact a rather minor modification of the output-activation mechanism (Mechanism A). For all these reasons the discussed experimental study does not contradict Mechanism C.

As it was discussed above, in intact skeletal muscle the maximal VO_2 is 2-5(-7) times greater than in isolated mitochondria [47,13,50]. On the other hand, Mootha and co workers [75] reported that the exercising dog heart and pig heart use only up to 80-90 % of the maximal oxidative phosphorylation capacity in isolated heart mitochondria. However, the maximal oxygen consumption in isolated mitochondria was measured at saturating concentrations of ADP, P_i, oxygen and respiratory substrates. On the other hand, [ADP] in intact heart in vivo is around the K_m value of oxidative phosphorylation for ADP and is essentially constant even during transition to very high work intensities [42,43]. Also [P_i] is constant and not saturating [42]. Furthermore, it is very likely that at high work intensities in intact heart oxygen and respiratory substrate supply by blood becomes limiting for oxygen consumption and ATP synthesis by oxidative phosphorylation. Taken together, the above

properties of the system in vivo suggest that the 'true' (potential) capacity of oxidative phosphorylation in intact heart may be significantly greater than in isolated mitochondria, in accordance with the idea of parallel activation (Mechanism C). It is also possible that the maximal oxygen consumption by heart is determined by the maximal ATPase activity and not by the capacity of oxidative phosphorylation. Generally, the discussed experimental data at least do not exclude the idea of parallel activation, and even seem to support this idea.

It has been also postulated that in quadrupeds the whole animal maximal sustained oxygen uptake is similar, when recalculated for the mitochondria volume, to the maximal oxygen uptake in isolated mitochondria [76,91]. However, a detailed analysis of the experimental data presented in [76] and [91] demonstrates that this conclusion is not so straightforward [45]. VO_2 per mitochondria volume during whole body spontaneous exercise (but not in electrically-stimulated muscle) in quadrupeds is almost twice greater than in isolated mitochondria incubated with the 'physiological' substrate (product of glycolysis): pyruvate [76,91] (succinate and, especially, cytochrome c are not physiological substrates of oxidative phosphorylation in skeletal muscle; with both substrates the VO_{2max} is overestimated, because succinate is an FAD-related substrate that 'omits' complex I and the 'key', rate-controlling TCA cycle dehydrogenases, while reduced cytochrome c is a substrate of cytochrome oxidase, the activity of which is much greater than the activity of entire oxidative phosphorylation – under physiological conditions cytochrome c is reduced in 20 – 30 % and not in 100 %). If it is taken into account that isolated mitochondria are incubated with saturating [ADP], [O_2] and [respiratory substrates] one can estimate that the 'true' capacity of oxidative phosphorylation in quadrupeds during whole body exercise is at least twice greater than in isolated mitochondria. Additionally, the maximal respiration rate in quadrupeds may be limited by the maximal activity of ATPases (especially actomyosin-ATPase) or by oxygen delivery to working muscles (in [76] the authors assumed that the maximal capacity of oxidative phosphorylation in working muscles corresponds to the maximal capacity in isolated mitochondria, which is not true if the parallel activation mechanism is correct; in such a case their conclusion that in quadrupeds oxygen supply to muscles is not limiting for VO_2 may be false). It is also evident (Table 1 in [91]) that VO_{2max} per mitochondria volume in quadrupeds is at least 3 times greater during spontaneous whole body exercise than during single muscle electrically-stimulated exercise. This experimental finding agrees very well with the proposal (see above) that

parallel activation is much more intensive in neurally stimulated muscle than in electrically stimulated muscle.

It seems that the (almost) perfect stability of metabolite (free ADP, PCr, P_i, NADH) concentrations (changes by less than 10 %) discussed above takes place in intact heart in vivo, but not in perfused heart, where some significant changes (by 10 - 300 %) in metabolite concentrations during work transitions are usually observed [77,92,93,78]. Additionally, the extent of these changes depends on respiratory substrates and hormones present in the perfusion medium [77]. However, in most cases these changes are too small to be reconciled with the Mechanism A or B. It has been proposed recently [18] that in perfused heart the parallel activation is disturbed: ATP supply is directly activated slightly less than ATP usage. Therefore, this situation seems to be similar to that prevailing in skeletal muscle, where the homeostasis of metabolite concentrations seems to be greater in muscle in vivo than in perfused muscle. The cited papers concerning the perfused heart suggest an important role of hormones in the stability of metabolite concentrations, and therefore in the parallel activation. Summing up, it can be postulated that in perfused heart both Mechanism C and Mechanism B (or A) contribute to the regulation of oxidative phosphorylation (like in oxidative skeletal muscle). Generally, different intensities of parallel activation are able to account for the variability of the kinetic properties of oxidative phosphorylation in heart and skeletal muscle.

Saks and co workers postulated in several papers (see e.g. [8]) that in skinned fibers without creatine there are great ADP diffusion gradients (due to e.g., a spatial arrangement of oxidative phosphorylation units) and creatine kinase (CK) is significantly displaced from thermodynamic equilibrium. However, there is no direct evidence that such great diffusion gradients/displacement of CK from equilibrium take place in intact muscle where there is no unstirred layer, as in skinned fibers, and the creatine shuttle overcomes the possible ADP diffusion limitations. Computer simulations assuming the each-step-activation mechanism (Mechanism C), but not involving ADP diffusion gradients/CK disequilibrium are able to reproduce very well the kinetic behavior of oxidative phosphorylation in intact skeletal muscle [15,30] and heart [14,18]. It was demonstrated in the experimental way that in skeletal muscle with creatine kinase knockout it is ADP and not Cr that directly activates mitochondria [94]. Nevertheless, the studies by Saks and co-workers are important to demonstrate the role of the creatine kinase system (CK + PCr + Cr) in overcoming possible ADP diffusion limitations and to reveal the structural and functional architecture of oxidative phosphorylation

enzyme complexes (miCK, porine pore, ATP/ADP carrier). However, structural organization (e.g., spatial arrangement of oxidative phosphorylation complexes) does not automatically imply kinetic compartmentalization in intact muscle in vivo, although such a compartmentalization may take place in skinned fibres, especially in the absence of Cr.

Also the mechanistic-ultrasensitivity mechanism (Mechanism D) seems to contain some truth in it. Namely, it was postulated that while the mechanistic dependence of oxygen consumption on [ADP] is near-hyperbolic (first-order at low ADP concentrations), the mechanistic dependence of the oxidative ATP production on [ADP] is near second-order [13] – the difference is due to proton leak that is responsible for over 50 % of oxygen consumption in resting skeletal muscle [79,80]. This fact could explain why in some isolated mitochondria where the proton leak is for some reasons low the mechanistic VO_2-[ADP] relationship is near second-order [5]. Therefore, both the mechanistic-ultrasensitivity mechanism (Mechanism D) and CK-disequilibrium mechanism (Mechanism E) seem to point at some important kinetic aspects of muscle energetics.

Of course, the choice of the above 16 experimental results as a reference point for the estimation of different mechanisms of the regulation of oxidative phosphorylation is to some extent arbitrary. This list can be extended, especially in the future, when new experimental data will become available. The author's intention was to select experimental results that are most important for muscle energetics. They concern mostly the biochemical and cellular level of organization; the only analyzed variable on the whole body level is the pulmonary oxygen consumption that is assumed to reflect pretty well the oxygen consumption at the skeletal muscle level. Nevertheless, presently a model of oxygen transport to working skeletal muscle is being developed and therefore this problem will be analyzed more explicitly in the future.

Whereas in many cases a good quantitative agreement between the model predictions and experimental results is observed, in several other cases the predictions agree with results only semi-quantitatively. However, first, every computer model is only a simplified and approximate representation of the reality. Second, frequently experimental results coming from different laboratories differ quite significantly. For this reason a good model should rather reflect semi-quantitatively different experiments, than strictly quantitatively one particular experiment. The discussed model based on Mechanism C is able to account for much more properties of the system than other models based on other mechanisms (see above and below) and therefore, in the author's

opinion, it offers at the present stage the best approximation of the reality. This does not mean, however, that the model is in any way perfect and that it cannot (and should not) be further tested, extended and modified.

One may address the question how independent of each other are the experimental results used in Table 2 and in the above discussion. For instance, is the fact that $[P_i]$ in slowly beating heart is in the millimolar range independent of the fact that $[P_i]$ is relatively stable during low-to-high work transition? However, one can easily imagine a theoretical situation where $[P_i]$ is very low and at the same time constant. On the other hand, the results 1-16 are of course related to each other if we look at them from the point of view of the parallel activation (each-step activation) mechanism, because they can be expelaind just by this mechanism.

Other theoretical studies

Some other computer models of oxidative phosphorylation were developed recently and used for theoretical studies concerning the regulation of this process; some of them have been already discussed above. Generally, the models represent particular mechanisms (A – E) distinguished in the present paper. Therefore, confrontation of the theoretical predictions made using the models with experimental data may be useful for the discussion concerning the mechanisms of the regulation of oxidative phosphorylation.

Beard [95] developed a model of oxidative phosphorylation in isolated mitochondria based on the experimental data presented in [86]. Within this model all reactions are described as being thermodynamically reversible, while in the Korzeniewski's model only the reactions that are reversible under physiological conditions (complex I, complex III, ATP synthase, ATP/ADP carrier and phosphate carrier, but not ATP usage, substrate dehydrogenation and cytochrome oxidase) are described by equations that fulfill thermodynamic requirements. The Beard's model contains an explicit description of the ion transport across the inner mitochondrial membrane that determines the relationship between ΔpH and $\Delta\Psi$, and therefore is more mechanistic in this aspect than the Korzeniewski's model. Within the model ΔpH is very close to zero and complex III is activated by P_i. The model is able to account for the increase in $\Delta\Psi$ when [P_i] increases from zero to physiological values [86]. However, as it was discussed above, the interpretation of the experimental data presented in [86] is not clear and the model developed by Beard relays on the validity of this interpretation. Generally, the validation of this model by comparison with experimental data is rather limited and the model predictions contradict many experimental studies, because in most experiments ΔpH is much greater than 0 (see the discussion above). This model represents the mechanism A (output activation) supplemented with the additional activation of complex III by P_i. Within the model an increase in VO_2 is accompanied by a significant increase in [P_i] and/or [ADP] and therefore this model cannot account for the prefect (or almost perfect) stability of metabolite concentrations in intact heart in vivo [42]. The recent version of this model for the intact heart [96] contains some unphysiological assumptions present in the model developed by Vendelin, Saks and co-workers (see the discussion above), namely: a very high (close to 100 %) contribution of proton leak to VO_2 and a very low [P_i] (a few μM) at low work intensity in heart, and a huge (by about three orders of magnitude) increase in [P_i] during low-to-high work

transition. Regardless if [P_i] in [42] is perfectly constant or slightly increases, certainly it does not change by several orders of magnitude, what Beard's model assumes.

The comprehensive model of oxidative phosphorylation, tricarboxic acid cycle and ion transport in cardiac myocytes developed by Cortassa and co-workers [97,98] represents the mechanism B (input/output activation). This model involves the activation by calcium ions of tricarboxic acid dehydrogenases during muscle contraction, and thus a direct activation of NADH production. The authors used their model to study in silico the time course of [NADH] in isolated trabeculae during low-to-high and high-to-low work transition (caused by varying electrical stimulation frequency) [83,84]. They were able to reproduce qualitatively the undershoot in [NADH] during the low-to-high work transition and the overshoot in [NADH] during the opposite transition [97,98]. However, in the first version of the model [97], an 8-fold increase in stimulation frequency, and thus in ATP usage for contraction and calcium transport, was accompanied by only a very small relative increase in the respiration rate (by 10-20 %, depending on assumptions). On the other hand, in the extended version of the model [98] the maximal NADH undershoot was equal to less than 2 % (in relation to the initial value) during transition from 0.25 Hz to 2 Hz stimulation frequency (Fig. 8 in [98]), whereas in experiments carried out in the isolated trabeculae system this undershoot equals almost 40 % [83,84] (see also Fig. 8 in [98]). This means a 20-fold difference between model predictions and experimental data concerning one of the most crucial variables in the system. The agreement between the simulated and measured dependence of the developed force and oxygen consumption, and stimulation frequency is also rather poor. Additionally, this model predicts very significant changes in [PCr] during transition between different stimulation frequencies and therefore cannot account for the prefect constancy of [PCr] in instant heart in vivo [42]. Next, the simulated half-transition time for PCr equals several hundreds of seconds (Fig. 5 in [98]), whereas this time equals a few seconds in perfused heart [74,78]. Finally, the VO_2 estimated from the initial rate of [PCr] decrease during transition from 0.25 Hz to 2 Hz stimulation frequency (Fig. 5 in [98]) (assumed to correspond to the rate of ATP turnover) equals about 0.15 mM/min (assuming that ATP/O ratio equals about 6), which is much smaller than VO_2 in intact heart (2-10 mM/min in dog heart [42] and even more in rat heart [77]) (unfortunately, the authors experss VO_2 in arbitrary units). On the other hand, the model developed by Korzeniewski and co-workers, involving Mechanism C (each-step-activation) is able to account at least semi-quantitatively for different properties (concerning steady-states and work transitions) (including

changes in VO_2 , ADP, PCr and NADH) in intact heart in vivo, in perfused heart and in isolated trabeculae [14,18].

Zhou and co-workers developed a model of the energetic system in heart cells in which a smaller emphasize is put on oxidative phosphorylation, but a greater emphasize is put on the substrate dehydrogenation (NADH-supplying system comprising TCA cycle, glycolysis, fatty acids β-oxidation, glucose an fatty acid transport and so on) [99,100]. When this model was supplemented with the parallel activation of different steps (oxidative phosphorylation and several NADH-supplying processes), a good agreement between simulated and experimentally measured reaction rates and metabolite concentration during low-to-high work transition in heart was encountered [101]. Therefore, this model, representing Mechanism C, certainly confirms and extends the idea of the direct parallel activation of different steps during elevated workload.

The computer model developed by Vendelin, Saks and co-workers [6,7], involving significant ADP diffusion gradients and displacement of CK from equilibrium, was discussed above in the context of the mechanism E (CK disequilibrium). It was demonstrated that this model/mechanism cannot explain most of the experimental results discussed in the present article. The discussed model is a little different from other models, because it involves a spatial arrangement of oxidative phosphorylation complexes. However, the predictions made by this model, for instance those concerning metabolic stability, can be compared with the predictions made by other models.

Generally, the theoretical predictions performed by different models that do not involve the each-step-activation mechanism demonstrate that several experimental data cannot be accounted for when the models used for simulations are based on other mechanisms. This fact further supports the idea of parallel activation.

However, it must be very clearly stated, that the above-discussed models of oxidative phosphorylation should not be identified with the mechanisms A-E. In principle, each mechanism can be ‘implemented’ in each model. For instance, the model developed by Korzeniewski and co-workers was used for studying the kinetic effect of mechanisms A – D [12,14]. On the other hand, the each-step-activation mechanism could be easily introduced to the models developed by Beard, Cortassa and co-workers as well as Vendelin, Saks and co-workers, just by assuming that all enzymes in the system are directly activated in parallel during low-to-high work transition. In fact, all these models (including also the model developed by Zhou and co-workers) are in principle quite similar to the

original model developed by Korzeniewski and co-workers. One could venture to say that the most important differences in their predictions result just from the assumed mechanisms of the regulation of oxidative phosphorylation.

Predicting new phenomena in biology

Of course the main criticism that may be addressed to the each-step-activation mechanism is that the physical nature of the factor(s)/mechanism(s) X responsible for the direct parallel activation of (particular components of) the oxidative phosphorylation system during low-to-high work transition remains unclear. Several evidences suggest some involvement of Ca^{2+} ions. For instance, calcium ions seem to activate ATP synthase in isolated mitochondria [102,103]. However, it is not certain if a high cytosolic and/or mitochondrial calcium concentration is the (only) factor looked for. Because high constant external [Ca^{2+}] only moderately activates isolated mitochondria [104,105] it has been postulated [16,17] that the relevant factor may be the frequency of calcium oscillations. This frequency could be integrated over time by some protein, analogous to calmodulin, causing protein phosphorylation/dephosphorylation, that is lost or inactivated during isolation of mitochondria. Recent studies show that Ca^{2+} ions are tunneled from sarcoplasmic reticulum to mitochondria in skeletal muscle [106]. On the other hand, the fact that ruthenium red, an inhibitor of Ca^{2+} uptake by mitochondria, only slightly lengthens the low-to-high work transition, but does not affect much the steady-state metabolite concentrations [107] suggests that either this inhibitor only delays but does not block calcium entry into mitochondria, or there exists another factor/mechanism that acts in parallel with calcium ions. There is also a possibility that, while TCA cycle dehydrogenases are activated by matrix calcium, the oxidative phosphorylation complexes, being transmembrane proteins located in the inner mitochondrial membrane, are activated by cytosolic calcium and therefore do not need the Ca^{2+} transport to the matrix for their activation.

One of the ways of heart stimulation (apart from neural and humoral stimulation) is mechanical stimulation through the Frank-Starling mechanism [2]. Due to this mechanism an increased preload pressure increases stroke volume and thus oxygen consumption [108]. It is not clear whether this mechanical stimulation is associated with an increase in cytosolic calcium concentration and/or with (almost) perfect metabolite stability (good metabolite stability was observed in electrically and humorally stimulated heart). However, at least in mammals, the central (neural) stimulation seems to play the dominant role in the stimulation of heart during e.g. physical exercise [109,110], and under physiological conditions mechanical stimulation always accompanies neural/humoral stimulation.

Several targets of the parallel activation in the substrate dehydrogenation block are known. It is widely recognized that Ca^{2+} ions activate TCA cycle dehydrogenases and glycogen phosphatase [9,10]. There are also strong evidences that skeletal muscle contraction causes a AMP- and PKA-mediated activation of fatty acid oxidation and glucose uptake [111].

Of course, computer simulations cannot by themselves reveal the physical nature of factor X. Many mechanisms different than the increase in the calcium ion concentration/oscillation frequency causing protein phosphorylation/dephosphorylation may be considered. For instance Halestrap proposed that changes in mitochondria volume and thus protein density serve as the regulatory factor [112]. Another possibility is an association of respiratory complexes in supercomplexes [113,114]. Therefore, of course, if the each-step-activation mechanism appears to be correct, it may be based on some factor/mechanism that is completely independent on calcium ions.

In the case of glycolysis it was also postulated that the regulation of this process in skeletal muscle by intermediate metabolites: ADP, AMP, ATP, P_i, NADH and NAD^+ (plus activation of glycogen phosphatase by calcium ions) is not sufficient to account for the kinetic behavior of this pathway in vivo [115] (for instance, ATP supply by anaerobic glycolysis is not significantly activated during anoxia and muscle recovery, when [ADP], [AMP] and [P_i] are elevated). In particular, it has been demonstrated that a direct activation of glycolysis by some external cytosolic factor is necessary to explain the large increase in the glycolytic flux taking place during transition from rest to heavy exercise in skeletal muscle [116]. Finally, Fell and Thomas paid attention to the fact that in many cases the glycolytic metabolite concentrations change little when the flux increases many times and postulated that the activity of different glycolytic enzymes is modulated in parallel by some unknown factor [24].

Nevertheless, the exact factor/mechanism that directly activates all oxidative phosphorylation complexes during muscle contraction still awaits to be discovered in the experimental way. This prevents many biologists (most experimentalists and many theoreticians) to believe in the each-step-activation mechanism. They argue that any explanation of the behavior of a considered system cannot be based on some unknown (not discovered) mechanism. This conservatism prevailing in biology is in a great contrast with the situations taking place in physics. It is very common in physics that some objects/phenomena are first predicted theoretically and then discovered experimentally; the microwave background radiation, gravitational lens, positron (anti-particle of electron), quark t and dilatation of time may serve as just a few examples. In fact, in the modern physics about a half of new discoveries

is due to unexpected findings, and the remaining half is due to testing theoretical predictions. If well tested theories predict the existence of some new phenomena, experimental physicists do not complain, by try to test such prediction in laboratories. In my opinion the time has come that this should be the case also in biology.

Generally, there are two opposite philosophies of modeling biochemical systems. Due to the first philosophy, a model should be always built bottom-up: experimental data obtained in vitro should be collected from different positions in the literature, put together and integrated as a computer model. If predictions of such a model do not agree with some experimental data concerning the macroscopic (high level) behavior of the modeled system, the discrepancies should be ignored as not important or temporary difficulties. Only the known elements/mechanisms/interactions may be included in the model. Most theoreticians prefer this way of model building. However, this is a very optimistic philosophy of modeling, because many enzyme properties in vitro differ very significantly from those in vivo, it is extremely difficult to study in vitro the properties of e.g. membrane complexes (especially oxidative phosphorylation complexes) and it is always possible that some yet undiscovered phenomena are present in the system.

Due to the second philosophy of biochemical systems modeling, a model should be of course to some extent based on the in vitro data concerning particular enzymes, but the priority is given to the agreement of the model with the macroscopic properties of the system. Due to this philosophy as simple as possible kinetic description of enzymes should be applied (especially in the case of the enzymes working near the thermodynamic equilibrium, where a detailed kinetics does not matter, because the forward reaction rate is similar to the backward reaction rate and therefore the net rate may be significantly changed by tiny changes in metabolite concentrations), and the set of enzyme kinetics should be tested by comparison of model predictions with the system behavior on the macroscopic level (e.g. of whole isolated mitochondria). To be reliable, any model should be tested for a possibly broad range (and not just a few) sets of parameters and system properties. If, under some conditions (e.g. in intact muscle), such a thoroughly validated model cannot account for some properties of the system, a new unknown element (factor, mechanism) can be introduced. Of course, it must be very carefully tested if the model supplemented with such new element is able to reproduce a possibly broad range of different system properties available in the literature. I am decidedly an advocate of such a philosophy of modeling.

In my opinion, the fact that factor/mechanism X has not been discovered yet, regarded by many people as the main disadvantage of the each-step-activation mechanism, may be equally well considered as a great challenge for the future experimental studies. Computer modeling and the each-step-activation idea offer a unique opportunity to predict the existence of some new, still undiscovered phenomena: this would be an exciting and novel achievement in biology. Of course, the discussed theoretical prediction may appear to be true or false. Only the future experiments can ultimately resolve this problem.

Conclusions

In the present polemic article it is demonstrated that the each-step-activation (parallel activation) mechanism of the regulation of oxidative phosphorylation during elevated energy demand in skeletal muscle, heart and other tissues, due to which all oxidative phosphorylation complexes are directly activated by some cytosolic factor/mechanism X in parallel with the activation of ATP usage and substrate dehydrogenation, is able to explain much more experimental data on the biochemical, cellular and physiological level than other mechanisms proposed in the literature. The broadly validated kinetic model of oxidative phosphorylation appeared to be a very useful tool for numerous in silico studies that decidedly support the idea of each-step activation. Because the exact physical nature of the factor/mechanism X is still unknown, this idea offers a unique opportunity to predict in the theoretical way the existence of some new phenomena that were not discovered yet in the experimental way. Such a prediction, if it appears to be correct, would be a novel and exciting achievement in Systems Biology.

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TABLES

Table 1. Changes in free [ADP] and oxygen consumption during transition from resting or low-work state to intensive-work state in different organisms and muscle types.

organism/muscle	increase in VO_2 (times)	increase in [ADP] (times)	references
rat gastrocnemius	10	2 - 2.5	[38,22]
dog gastrocnemius	18	5 - 6	[36,22]
human forearm flexor	>10	4	[5]
greyhound biceps femoris	200	4 - 5	[37,22]
thoroughbred leg muscle	>60	~ 2	[40,22]
human calf muscle	15	5	[39,22]
trained rat hindlimb muscle	30	2	[69]
human soleus	> 20	< 1.5	[35]
insect flight muscle	600	2	[41,22]
dog heart (in vivo)	5	1 (no increase)	[42]

Table 2. Confrontation of different mechanisms of regulation of oxidative phosphorylation with experimental Results 1 - 16 described in the text. +, the mechanism can account for the result; ±, the mechanism can only partially explain the result; -, the mechanism cannot account for the result; ?, it is not clear if the mechanism can account for the result.

	Result:															
Mechanism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
(A) Output activation	-	-	+	-	+	-	-	-	+	-	-	-	+	-	-	-
(B) Input/output activation	-	±[c]	+	-	+	+	-	-	+	-	-	-	+	-	-	±
(C) Each-step activation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(D) Mechanistic ultrasensitivity	±[a]	-	-[d]	-	+	-	-	-	-	+	-	-	-	-	-	-
(E) CK disequilibrium	?[b]	-	+	-	-	-	-	-	-	?[e]	-	-	-	±[f]	-	-

a) not in heart where the phenomenological VO_2-[ADP] is of an infinite order (no changes in [ADP]);

b) not under physiologically-relevant assumptions, compare Result 4 and Result 5;

c) the direct activation of substrate dehydrogenation can only slightly elevate the maximal VO_2 because the flux control coefficient of substrate dehydrogenation over the oxygen consumption flux is low;

d) in some experiments the VO_2-[ADP] dependence in isolated mitochondria is steeper than first-order [5];

e) displacement of CK from thermodynamic equilibrium shortens $t_{1/2}$, but diffusion limitations are likely to lengthen $t_{1/2}$;

f) it has been shown [7] that diffusion limitations can modulate the relationship between VO_2 and [ADP];

Figure legends

Fig. 1. General scheme of oxidative phosphorylation in muscle. Enzymes/processes/metabolic blocks that are taken into account explicitly within the model of oxidative phosphorylation are shown. SH, respiratory substrates; e^-, electrons; cyt. c, cytochrome c. The outer membrane and the intermembrane space are not shown because they are not taken into account explicitly within the model (that are treated as a part of the cytosolic compartment).

Fig. 2. Schematic presentation of the phenomenological VO_2-[ADP] relationship in different skeletal muscles. The lowest curve corresponds also approximately to isolated mitochondria. The effect of muscle training is indicated.

Fig. 3. Schematic presentation of the phenomenological VO_2-[ADP] relationship in intact heart in vivo. The dependence for no parallel activation is shown; because of a lower total phosphate concentration than in skeletal muscle this dependence is very steep at the left end (see [14] for details).

Fig. 4. Simulated changes over time of [PCr] at rest, during work and during muscle recovery after exercise for three values of the characteristic decay time of the activation of oxidative phosphorylation: 0 s, 100 s and 300 s. Details are given in the text.

Fig. 5. Explanation of the PCr recovery overshoot. The activities (rate constants) of ATP usage and ATP production by oxidative phosphorylation at rest, during work and during muscle recovery after exercise are shown. It can be seen that the activation of oxidative phosphorylation is not switched off instantly after the termination of exercise, but slowly decays in the exponential way. Details are given in the text.

Fig. 1

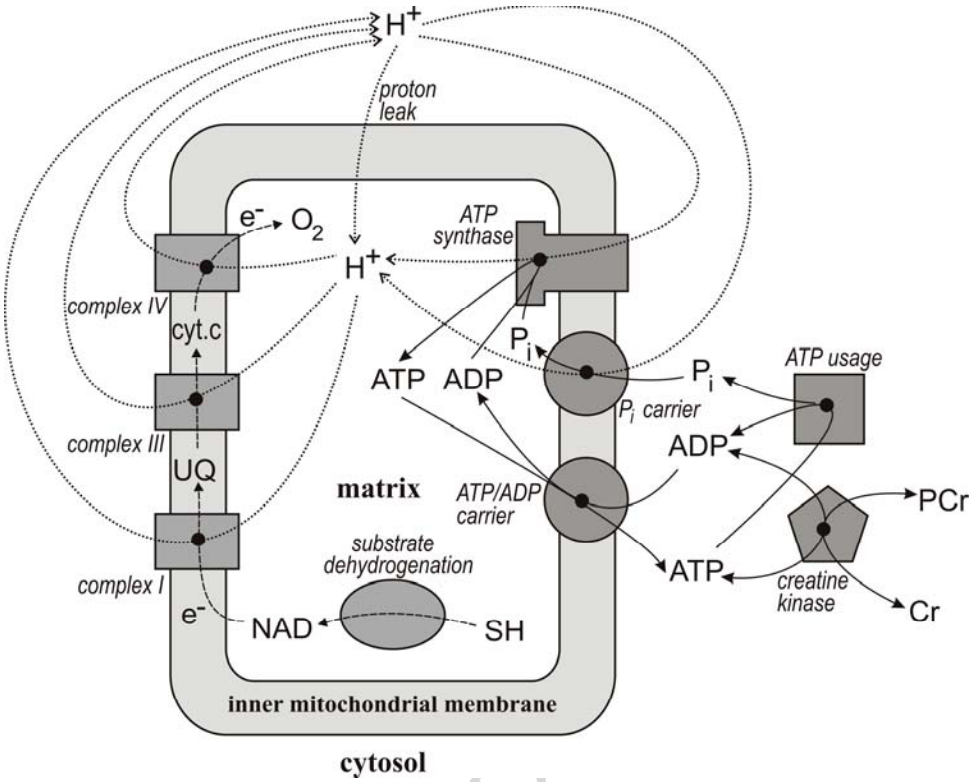

Fig. 2

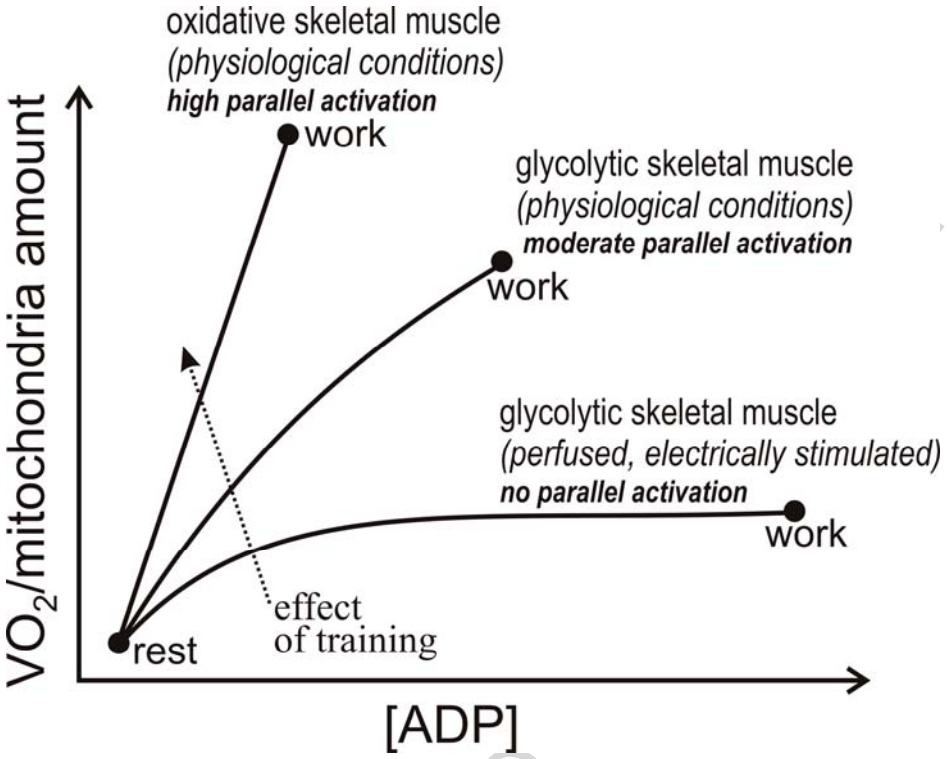

Fig. 3

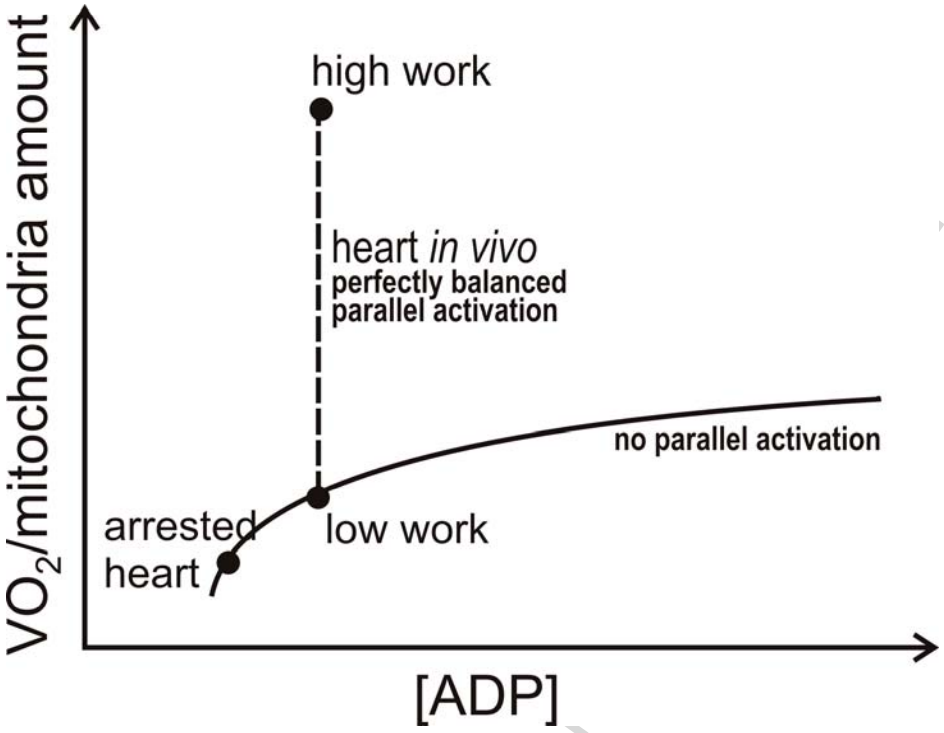

Fig. 4

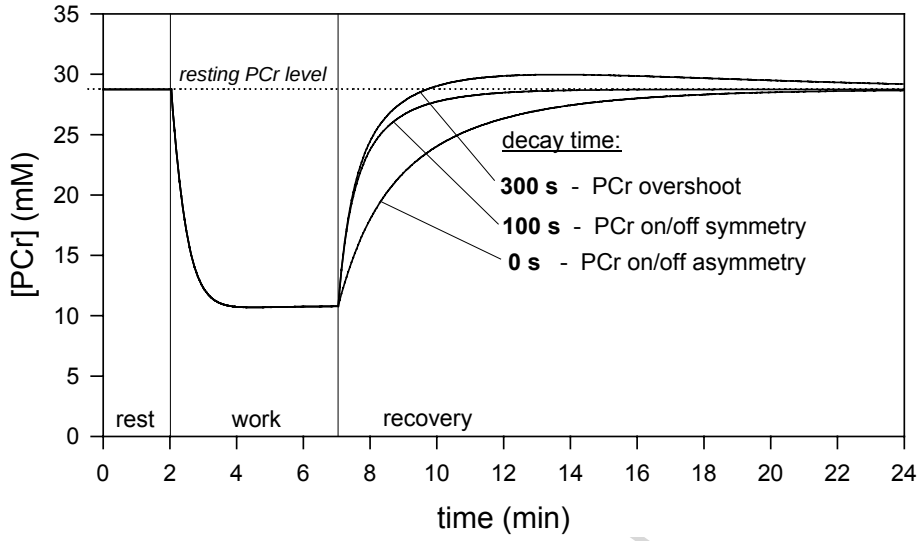

Fig. 5